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# DATA EVALUATION RECORD
# FISH LIFE-CYCLE TOXICITY TEST (MODIFIED)
## §72-4(a) & 72-5

1. **CHEMICAL:** Vinclozolin PC Code No.: 113201

2. **TEST MATERIAL:** Sodium Salt of Metabolite B of Vinclozolin Purity: 98.5%

3. **CITATION:**

Author: Zok, S.

Title: Modified Life Cycle Study with the Fathead Minnow (*Pimephales promelas*) and Vinclozolin in the Presence of its Metabolites B and E (Limit Test).

Study Completion Date: September 26, 2000

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Laboratory Report ID: 81F0055/985011

MRID No.: 45243703

DP Barcode: D270242

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## 6. STUDY PARAMETERS:

**Scientific Name of Test Organism:** *Pimephales promelas*

**Age of Test Organism:** Approximately 8 months old ($F_0$ generation)

**Definitive Test Duration:** 126 Days

**Study Method:** Flow-through

**Type of Concentrations:** Mean-measured

## 7. CONCLUSIONS:

In a modified fish life-cycle toxicity test, 8-month old fathead minnow (*Pimephales promelas*) were exposed for 112 Days under flow-through conditions to a mean analytically-determined concentration of 0.12 ± 0.025 mg total vinclozolin residues/L [vinclozolin, Metabolite B (acid), and Metabolite E (amide)]. Beginning on Day 78, embryos of the $F_1$-generation were isolated and exposed for 6 weeks (Day 126) under flow-through conditions to a mean analytically-determined concentration of 0.12 ± 0.032 mg total vinclozolin residues/L.

$F_0$-generation: There were no treatment-related effects on survival, terminal body weight or length, or histopathological changes in gonad development. The reproductive behavior of driving females to the brood hole was slightly statistically-reduced compared to controls. In addition, 2/15 pair of treated fish exhibited delayed egg production (not statistically evaluated). In both cases, since only one concentration was used, the importance of these findings could not be accurately assessed. The number of spawns/female was statistically-reduced compared to controls (14.7 for controls versus 6.73 for the test group); however, the number of eggs/spawn was notably higher in the treatment group (65.4 for controls versus 113.7 for the test group). Consequently, the number of eggs/female was only slightly lower in the test group (959 for controls versus 766 for the test group), with no statistical significance. Therefore, the biological significance of this finding could not be accurately assessed.

$F_1$-generation: There were no treatment-related effects on survival following the completion of hatch, the time to hatch or time to swim-up, terminal body weight or length, or histopathological changes in gonad development. In addition, no treatment-related signs of toxicity were observed. Hatch survival was statistically-reduced both at the start and end of hatch compared to controls (66.5% for controls versus 42.5% for the test group at the end of hatch). Since only four pairs/test group contributed to the production of the $F_1$-generation and since fertility rates in the $F_0$-generation showed high variability, it was concluded that the biological relevance of the lower survival until hatch finding can not be

evaluated since only one concentration group was available.

In conclusion, because only one test concentration was used in this study, neither a LOEL nor a definitive NOEL was established.

**This study is classified as SUPPLEMENTAL.** It is scientifically valid, but was performed under conditions that deviated substantially from recommended protocols. Although results do not meet guideline requirements; the information may be useful in a risk assessment.

## 8. ADEQUACY OF THE STUDY:

**A. Classification:** Supplemental

**B. Rationale:** This study deviated significantly from recommended protocols for both the fish early life-stage toxicity test [§72-4(a)] and the fish life-cycle toxicity test (§72-5), and, therefore, only provides supplemental data on the toxicity of vinclozolin on fathead minnow.

**C. Repairability:** N/A

## 9. GUIDELINE DEVIATIONS:

1. The study was conducted in accordance with OECD Principles of Good Laboratory Practice (Paris, 1981) and the GLP provisions of the Chemikallgesetz (FRG, 1990/1994).

2. A single nominal concentration level of 0.1 mg vinclozolin/L was used.

3. $F_0$-generation fish were 8 months old at study initiation.

4. $F_0$-generation fish that died during the study were replaced with fish from a concurrently-maintained reserve/replacement group.

5. $F_1$-generation fish were maintained for 6 weeks, instead of the recommended 8 weeks.

6. The test water was aerated during exposure for all groups.

7. Despite aeration, the dissolved oxygen content was only generally maintained at >60% saturation.

8. Aside from being bred in the testing facility, information regarding the source of the $F_0$-generation fish was not provided.

9. Lighting conditions during acclimation of the $F_0$-generation fish were not reported.

10. pH was generally maintained at 7.0 ± 0.5, which is slightly lower than the recommended range of 7.2 to 7.6 for this test species.

11. Measured test water hardness (210-250 mg/L as $CaCO_3$) was significantly higher than recommended levels (40-48 mg/L).

12. Alkalinity and conductance were apparently not measured.

13. The size and design of larval chambers did not follow guideline recommendations.

14. Flow rates were significantly higher than recommended rates; whereas, 100% replacement was achieved in 4.7 hours for the $F_0$-generation and 3.2 hours for the $F_1$-generation.

**10. SUBMISSION PURPOSE:** Re-registration

**11. MATERIALS AND METHODS:**

**A. Test Organisms**

| Guideline Criteria | Reported Information |
|---|---|
| **Species**<br>Prefer Sheepshead minnow (*Cyprinodon variegatus*) or Fathead minnow (*Pimephales promelas*). | Fathead minnow (*Pimephales promelas*) |
| **Source** | $F_0$-generation fish were bred in the testing facility. No further information was provided. |
| **Acclimation** | For 14 days prior to test initiation, fish were maintained at 21-23°C. Lighting conditions were not described. The pH was reduced "stepwise" from 8.0 to 6.9-7.0. No mortality was observed during this period. |
| **Age at beginning of test**<br>Embryos, 2 to 24 hours old | Approximately 8 months |

| Guideline Criteria | Reported Information |
|---|---|
| **Feeding**<br>Fish should be fed at least twice daily and should not be fed for at least 24 hours prior to test termination. | During acclimation: TetraMin and frozen or newly hatched brine shrimp larvae (*artemia naupli*).<br><br>Throughout exposure of $F_0$ generation: 1:1 mix of TetraMin and Kronen Fish Aminostart twice daily on workdays and once daily on weekends at 2% of the fish body weight. Newly hatched brine shrimp (*artemia naupli*) were also offered (not further specified). Feeding was discontinued 1 day prior to test termination.<br><br>Throughout exposure of $F_1$ generation: Newly hatched larvae were fed Microplan beginning 2 days after start of hatch. Beginning 9 days after start of hatch, fish additionally received fine-milled Kronen Fish Aminostart and TetraMin *ad libitum* in 1-3 portions daily until 1 day before test termination. |
| **Embryo Exposure (4 to 5 Days)**<br>Embryos (≤24 hours old) from at least 3 separate spawns should be randomly distributed to embryo cups.<br><br>A minimum of 50 embryos (≤24 hrs old) per replicate cup, 4 cups per treatment should be used.<br><br>Parameters measured:<br>· Survival of embryos<br>· Time required to hatch<br>· Hatching success<br>· Survival of fry for 4 weeks<br><br>Dead and fungused embryos should be | N/A. Exposure began when $F_0$-generation fish were 8 months old. |

| Guideline Criteria | Reported Information |
|---|---|
| counted and removed daily. | |
| **Larval-Juvenile Exposure (From Hatch to 8 Weeks)**<br>After hatching, each group of larvae is randomly reduced to a minimum of 25 fish and released in replicate larval growth chambers. The random selection must include any fish that are lethargic or deformed.<br><br>Parameters measured:<br>Fish survival (determined by counting the number of live fish in each replicate growth chamber weekly).<br><br>Total lengths (mm) of all fish at 4 and 8 weeks after hatching. | N/A |
| **Juvenile-Adult Exposure (From 8 weeks posthatch to the end of the spawning phase [32-40 weeks])**<br>At 20-24 weeks after hatching, mature fish are placed in a spawning tank of the same concentration (4 males and 4 females randomly chosen and assigned). The spawning tank is divided into 4 individual spawning chambers with appropriate spawning substrates.<br><br>The substrates are examined daily and embryos removed, counted, and recorded separately for each pair. | Eight-month old fish (sex not determined) were divided into four replicates of 20 fish per treatment level and exposed for 34 days.<br>At Day 34, sexes were determined and weight and length were recorded. The number of fish were then reduced to 16 pairs per treatment level: one pair/cage, four cages/aquaria, and four aquaria per treatment level. Each cage contained a plastic half tube for deposition of eggs. Remaining fish were sacrificed and the gonads were examined histologically.<br><br>During the 112-day $F_0$-exposure, an additional 16 fish/test group were exposed in one aquaria and served as a reserve/replacement group.<br><br>Daily observations included mortality, changes in appearance and behavior, |

| Guideline Criteria | Reported Information |
|---|---|
| For fathead minnow, adult exposure should be terminated when no spawning occurs for one week. For sheepshead minnow, testing should be terminated after spawning is observed for 2 weeks. | coloration of the male, reproduction behavior (twice daily), egg number, and the fertilization rate of the eggs laid.<br><br>The $F_0$-generation pairs were observed over 11 weeks (Days 34-112). At Day 112, the fish were sacrificed, terminal lengths and weights were recorded, and the gonads were examined histologically. |
| **Second Generation Embryo Exposure (4 to 5 days)**<br>50 embryos from each conc. level are randomly selected and transferred to incubation cups for hatch. Use the same test procedures as those for parental generation.<br><br>Embryos not selected are discarded. | $F_1$-generation exposure began on Day 78, with 25 eggs/cup, two egg cups/replicate aquarium, and four replicate aquaria/treatment level. Eggs from Day 78 (available from four to five pairs/group) were counted per pair, combined, and randomly distributed.<br><br>To serve as a viability control, an additional egg cup containing 145 eggs (source not specified, presumably from exposed fish) was maintained for the first 24 hours in one of the control replicate aquaria. Embryo survival was assessed after 1 day. The study author reported, "The exposure of the egg cup in the same test vessel as the control group was considered to have no influence, since the eggs of both groups had no direct contact, the oxygen consumption at this developmental stage is negligible and no pollution of the test water by the test organisms has to be considered" (p. 25).<br><br>Daily observations included the number of hatched larvae, survival of fry (estimated), and changes in appearance and behavior/deformations. The start and end of hatching were also recorded, and once weekly, the number of surviving fish was |

| Guideline Criteria | Reported Information |
|---|---|
| | verified using photography. |
| **Second Generation Larval-Juvenile Exposure (From Hatch to 4-8 weeks)**<br>After hatching, 25 larvae are released in each growth chambers (2 chambers per treatment). | Approximately 1 week after hatch, the surviving larvae were released into the aquarium; fry were not thinned. |
| Each group of 2nd generation fish is terminated 8 weeks after hatching. | Fry were sacrificed on Day 126, 6 weeks after hatching. |
| Fish are blotted, weighed, and measured before being discarded. | Length and weight were measured, and gonads were histopathologically examined. |

**B. Test System**

| Guideline Criteria | Reported Information |
|---|---|
| **Test Water**<br>Fathead Minnow<br>1. Reconstituted water or water from unpolluted well or spring (sterilized and tested for pollutants). | 1. Non-chlorinated drinking water obtained from the city of Frankenthal was purified through a charcoal filter and aerated. Routine analysis concluded that the tap water was reportedly free of heavy metals and impurities. |
| 2. Hardness of 40 to 48 mg/L as $CaCO_3$ and pH of 7.2 to 7.6. | 2. Hardness was approximately 210-250 mg/L as $CaCO_3$ and pH was generally maintained at 7.0 ± 0.5, with peaks of 8.3 in the $F_0$-generation and 7.9 in the $F_1$-generation. |
| **Test Temperature**<br>Fathead: 25°C and should not remain outside the range of 24 to 26°C for more than 48 hours.<br><br>Sheepshead: 30°C. | Generally 24 ± 1°C; on four occasions, temperatures of 22 or 26°C were measured in single replicates between 80 and 98 days in the $F_1$-generation aquaria. |

| Guideline Criteria | Reported Information |
|---|---|
| **Photoperiod**<br>16-hour light/8-hour dark.<br><br>Light intensity of 10-100 lumens at water surface. | $F_0$-generation: Through Day 56, 16-hours light/8-hours dark, 100-200 Lux (lumen/$m^2$). Thereafter, 17-hours light/8-hours dark, 500-800 Lux.<br>$F_1$-generation: 16-hours light/8-hours dark, 100-200 Lux. |
| **Dosing Apparatus**<br>1. Intermittent flow proportional diluters or continuous flow serial diluters.<br>2. A minimum of 5 toxicant concentrations with a dilution factor $\leq$0.5.<br>3. One control should be used. | 1. A continuous-flow diluter was used.<br><br>2. 1 toxicant concentration was used.<br><br>3. One negative control was used. |
| **Toxicant Mixing**<br>1. Mixing chamber recommended but not required.<br><br>2. Test solution completely mixed before introduction into the test system (aeration should not be used for mixing).<br><br>3. Flow splitting accuracy must be within 10% and periodically checked. | 1. A mixing chamber was used.<br><br>2. Yes<br><br>3. Maximum deviation reportedly less than 10%. |
| **Exposure System/Test Vessels**<br>Exposure tanks should be all glass or glass with a plastic or stainless steel frame (30.5 x 30.5 x 91.4 cm or 30.5 x 30.5 x 61 cm for fathead, and 45 x 90 x 26 cm for sheepshead).<br><br>Larval chambers should have glass bottoms and drains that allow water to be drawn down to 3 cm. | Prior to thinning into pairs (on Day 34), $F_0$-generation fish were exposed in glass aquaria measuring 60 x 30 x 30 cm (47-L total volume). After thinning into pairs, fish were maintained in plastic-coated stainless-steel lattice cages (four per aquaria), each measuring 28 x 13.5 x 26.5 cm.<br><br>$F_1$-generation fish were exposed in glass aquaria measuring 44 x 10.5 x 25 cm (8-L total volume). |

| Guideline Criteria | Reported Information |
|---|---|
| Test water depth in adult tanks and larval chambers should be a minimum of 15 cm. | Test water depth in adult and larval chambers was 26 and 18 cm, respectively. |
| **Embryo and Fry Chambers**<br>120 mL glass jars with bottoms replaced with 40 mesh stainless steel or nylon screen. Chambers can be oscillated vertically using rocker arm apparatus (2 rpm motor) or placed in separate chambers with self-starting siphons. | Glass cylinders, 6-cm diameter, 10-cm height (283 mL), with 0.3-mm stainless-steel mesh bottoms. The cylinders were moved slowly up and down with an eccentric shaft. |
| **Flow Rate**<br>Flow rates to adult tanks or larval chambers should provide 90% replacement in 8-12 hours, and maintain DO at above 75% of saturation. The toxicant level cannot drop below 20% with fish in the tank. | $F_0$-generation:<br>10 L/hour/aquarium, equivalent to 100% replacement in 4.7 hours and 5.1 volume replacements/day.<br><br>$F_1$-generation:<br>2.5 L/hour/aquarium, equivalent to 100% replacement in 3.2 hours and 7.5 volume replacements/day.<br><br>The DO was generally maintained above 60% of saturation; four exceptions were noted, with the lowest value of 49% measured in the $F_1$-generation on Day 92 due to a defect in the aeration tube. |
| **Aeration**<br>Dilution water should be aerated to insure dissolved oxygen concentrations at or near 100% saturation. Test tanks and embryo chambers should not be aerated. | Dilution water was aerated prior to entering the mixing chamber. In addition, test tanks were aerated from Day 6 on for the $F_0$-generation and from Day 88 on (1 week after start of hatch) for the $F_1$-generation. |

**C. Chemical System**

| Guideline Criteria | Reported Information |
|---|---|
| **Nominal Concentrations**<br>Minimum of 5 concentrations and a control, all replicated; plus solvent control if appropriate.<br><br>Toxicant conc. must be measured in one tank at each toxicant level every week. | 0 (negative control) and 0.1 mg/L<br><br>Toxicant concentration was measured from alternating replicate aquaria in each test group generally twice weekly: 1 day after the start of a new stock solution and on the last day of use before the stock solution was replaced. |
| **Other Variables**<br>1. DO must be measured at each conc. at least once a week.<br>2. Test water temp. must be recorded continuously.<br><br>3. Freshwater: A control and one conc. must be analyzed weekly for pH, alkalinity, hardness, and conductance.<br>Natural seawater: must maintain a constant salinity and not fluctuate more than 6% weekly; monthly pH range <0.8 pH units. | 1. DO measured in all replicate aquaria every 3 to 4 days.<br>2. Temperature measured in alternating replicate aquaria once daily, and measured continuously in both (control and test) reserve $F_0$-generation groups.<br>3. pH measured in all replicate aquaria daily. Water hardness was measured in the dilution water supply for each test group once weekly. Alkalinity and conductance were apparently not measured. |
| **Solvents**<br>Should not exceed 0.1 ml/L in a flow-through system. Acceptable solvents are: dimethylformamide, triethylene glycol, methanol, acetone, ethanol. | N/A |

**Comments**: The study author reported that vinclozolin is sparingly soluble in water, and is transformed to its metabolites B (acid) in a reversible reaction and E (amide) in an irreversible reaction. The reactions are dependent on the pH value. Therefore, the mean analytically-determined values of the test substance in the test water were generally below the range of ± 20% of the nominal concentration and showed relatively high variations.

It was reported that 0.1 mg/L vinclozolin was considered to be the highest concentration which can be tested under realistic conditions without the use of solvents. This test was considered to be a "limit test". Upon chemical analysis of the test waters, 3,5-dichloroaniline was also detected in low amounts (≤0.08 mg/L) in the treated group in both generations.

## 12. REPORTED RESULTS:

### A. General Results

| Guideline Criteria | Reported Information |
|---|---|
| **Quality assurance and GLP compliance statement were included in the report?** | Yes; however, this study was conducted in compliance with OECD Principles of Good Laboratory Practice (Paris, 1981) and the GLP provisions of the Chemikallgesetz (FRG, 1990/1994). |
| **Data Endpoints** must include:<br>· survival of $F_0$ and $F_1$ embryos, time required to hatch, and hatching success;<br>· survival and total length of $F_0$ fish at 4 and 8 weeks after hatching;<br>· weights and lengths of $F_1$ fish at 8 weeks;<br>· incidence of pathological or histological effects; and<br>· observations of other effects or clinical signs. | Data Endpoints included:<br>· survival of $F_1$ embryos, time required to hatch, and hatching success;<br>· survival and weights and lengths of $F_0$ fish at 34 and 112 Days after study initiation;<br>· weights and lengths of $F_1$ fish at 6 weeks;<br>· histologically examination of $F_0$ and $F_1$ gonads; and<br>· observations of other effects or clinical signs. |
| **Raw data included?** | Yes |

Concentration Results:

| Group | Mean analytically-determined concentration ± SD (mg/L)[a] | | | |
|---|---|---|---|---|
| | Metabolite B | Metabolite E | Vinclozolin | Sum |
| $F_0$-generation | 0.05 ± 0.015 | 0.008 ± 0.003 | 0.07 ± 0.02 | 0.12 ± 0.025 |
| $F_0$-reserve group | 0.06 ± 0.037 | 0.014 ± 0.005 | 0.06 ± 0.041 | 0.13 ± 0.077 |
| $F_1$-generation | 0.05 ± 0.016 | 0.009 ± 0.004 | 0.07 ± 0.024 | 0.12 ± 0.032 |

[a]The nominal concentration was 0.1 mg/L.

**$F_0$ Results:**

| Mean Measured Total Residues Conc. (mg/L) | Number Initially Exposed | % Survival Prior to Thinning (Day 34) | Number After Thinning (Day 34) | % Survival at Test Termination (Day 112) |
|---|---|---|---|---|
| Control | 80 | 98.8 | 16 pair | 87.5 |
| 0.12 ± 0.025 | 80 | 98.8 | 16 pair | 87.5 |

| Mean Measured Total Residues Conc. (mg/L) | Mean Total Length (cm) | | | Wet Weight (g) | | |
|---|---|---|---|---|---|---|
| | Day 34[a] | Day 112 (♂) | Day 112 (♀) | Day 34[a] | Day 112 (♂) | Day 112 (♀) |
| Control | 5.8 | 7.0 | 5.9 | 2.84 | 4.60 | 2.33 |
| 0.12 ± 0.025 | 5.8 | 6.9 | 6.0 | 2.68 | 4.41 | 2.61 |

[a]Not analyzed statistically, since histological results showed that males and females were not clearly distinguished at the time.

| Mean Measured Concentration (mg/L) | Number of Pairs Evaluated[a] | Total Number of Spawns | Total Number of Eggs | Number of Eggs/ Spawn | Number of Spawns/ Female | Number Eggs/ Female |
|---|---|---|---|---|---|---|
| Control | 12 | 176 | 11504 | 65.4 | 14.7 | 958.7 |
| 0.12 ± 0.025 | 15 | 101 | 11485 | 113.7 | 6.73** | 765.7 |

**Statistically-significant from control at p≤0.01.
[a]It was ultimately determined that 4/16 control pair and 1/16 test pair were male/male combinations.

Toxicity Observations: Reproduction behavior, including the coloration of the males, standing of the male in the brood hole, and the activity of driving the female to the spawning place, were observed twice daily on workdays. There was a slight statistically-significant (p≤0.05) decrease in the activity of driving the females to the brood hole in the

treated group (55% for controls versus 45% for the 0.12-mg/L group). The study author reported that since no historical data on this parameter are available and since only one concentration was tested, it is questionable whether this small difference is test substance-related or an arbitrary effect.
One pair from each group did not spawn. In addition, two of the 15 pair from the treatment group exhibited delayed egg production, defined as the time in which egg production started >3 weeks after egg production had started in the last control pair. The study author reported that this can not be interpreted as a substance-related effect, since only one test group is available and the number of pairs is comparably low.

The number of spawns/female was statistically ($p \leq 0.01$) reduced compared to controls, while the number of eggs/spawn was markedly higher in the treatment group. Consequently, the number of eggs/female was slightly lower in the test group, without statistical significance. Therefore, the study author reported that the biological significance of the lower number of spawns/female is questionable.

No treatment-related pathological changes were observed in gonads of fish sacrificed after 34 or 112 Days.

**$F_1$ Results:**

| Mean Measured Concentration (mg/L) | Initial Number of Embryos (Day 78) | Percent Normal Embryos After 24 Hours[a] | Percent Embryo Survival at Start of Hatch (Day 81)[b] | Percent Embryo/Larval Survival at End of Hatch (Day 84)[b] | Percent Survival at Study Termination (Day 126)[c] |
|---|---|---|---|---|---|
| Control | 200 | 91 | 84.0 | 66.5 | 69.2 |
| 0.12 ± 0.032 | 200 | 84 | 55.5** | 42.5** | 62.4 |

**Statistically significant from control at $p \leq 0.01$.
[a]Survival in the viability control group after 1 day was 88%.
[b]Relative to Day-78 embryos.
[c]Relative to Day-84 survivors.

| Mean Measured Concentration (mg/L) | Terminal Mean Length (mm) | Terminal Mean Wet Weight (g) |
|---|---|---|
| Control | 3.07 | 0.29 |
| | | |

| Mean Measured Concentration (mg/L) | Terminal Mean Length (mm) | Terminal Mean Wet Weight (g) |
|---|---|---|
| 0.12 ± 0.032 | 2.95 | 0.26 |

Toxicity Observations: No treatment-related effects on the time to hatch or swim-up time were observed. The start of hatch occurred on Days 81-82 for the control group and Day 82 for the test group. The end of hatch occurred on Days 83-84 for both groups, and swim-up was completed in all replicates on Day 85.

A statistically-significant ($p \leq 0.05$) reduction in hatch survival (both at the beginning and end) was observed. Once hatching was completed, no significant effect on overall (Days 84-126) survival of $F_1$-generation fish was observed. The study author reported that since only four pairs/test group contributed to the production of the $F_1$-generation and since fertility rates in the $F_0$-generation showed high variability, the lower survival rate at the start of the $F_1$-generation might be due to the normal variation between pairs and even between the spawnings of one pair. The study author concluded that the biological relevance of the lower survival until hatch finding can not be evaluated since only one concentration group is available and a concentration effect relationship can not be detected.

Abnormal behavior, toxic signs, and morphological abnormalities were observed daily beginning on Day 91. No treatment-related effects were observed. No treatment-related effects on terminal (Day 126) body weight or length were observed, and histopathological evaluation revealed no qualitative difference between the development of gonads of the test and control group.

**B. Statistical Results**

Statistical Method: The following parameters were subjected to statistical analyses: the $F_0$-generation wet body weight and length of surviving males and females at study end, $F_0$-generation reproduction behavior, $F_0$-generation egg data (number of eggs, number of spawns, number of fertile eggs, fertility rate, and mean clutch size), $F_1$-generation embryo survival Day 78-81 (start of exposure until beginning of hatch), $F_1$-generation embryo/sac fry survival Days 81-84 (beginning of hatch until termination of hatch), $F_1$-generation survival of larvae Days 78-84 (start of exposure until termination of hatch), $F_1$-generation survival Days 84-126 (termination of hatch until sacrifice), $F_1$-generation survival Days 78-126 (over complete exposure until sacrifice), $F_1$-generation wet body weight and length at sacrifice, and $F_1$-generation sex ratio.

Body weight and lengths were evaluated using Student's t-test (two-sided) for each sex.

Reproduction behavior, egg data, and the sex ratio were evaluated using the Wilcoxon-Test: one-sided for the behavioral parameters, the number of eggs per female, and the fertility rate per pair and two-sided for the number of clutches per pair, the mean clutch size, and the sex ratio. For the embryo, larvae, and fish survival, a pairwise comparison of the dose group with the control group was carried out via the log-rank test (one-sided).

| Biological Endpoint | NOEC (mg/L, total residues) | LOEC (mg/L, total residues) |
|---|---|---|
| $F_0$ 34-Day survival | 0.12 ± 0.025 | >0.12 ± 0.025 |
| $F_0$ test termination (Day 112) survival | 0.12 ± 0.025 | >0.12 ± 0.025 |
| $F_0$ test termination length (Males) | 0.12 ± 0.025 | >0.12 ± 0.025 |
| $F_0$ test termination length (Females) | 0.12 ± 0.025 | >0.12 ± 0.025 |
| $F_0$ test termination weight (Males) | 0.12 ± 0.025 | >0.12 ± 0.025 |
| $F_0$ test termination weight (Females) | 0.12 ± 0.025 | >0.12 ± 0.025 |
| $F_0$ # of spawns/female | Not determined | 0.12 ± 0.025 |
| $F_0$ # of eggs/female | 0.12 ± 0.025 | >0.12 ± 0.025 |
| $F_1$ hatching success (Day 84) | Not determined | 0.12 ± 0.032 |
| $F_1$ 6-week (Day 126) survival | 0.12 ± 0.032 | >0.12 ± 0.032 |
| $F_1$ 6-week length | 0.12 ± 0.032 | >0.12 ± 0.032 |
| $F_1$ 6-week weight | 0.12 ± 0.032 | >0.12 ± 0.032 |

**NOEC:** Not determined **LOEC:** 0.12 mg total residues/L **MATC:** Not determined

**13. REVIEWER'S STATISTICAL RESULTS:**

Statistical Method: One-tailed *t*-tests were used to assess treatment effects because only a one control and one treatment group were used. As a result, definitive NOEC, LOEC, and MATC estimates could not be determined. For sublethal effects and mortality data, visual analysis was used to assess adverse effects of treatment.

Statistically Significant Endpoints: Reductions were observed for number of egg clutches, embryo survival (at hatch beginning), larvae survival (at hatch termination), and survival from day 78 to test termination.

## 14. REVIEWER'S COMMENTS:

This study is classified as SUPPLEMENTAL. The study design deviated significantly from recommended protocols for both the fish early life-stage toxicity test [§72-4(a)] and the fish life-cycle toxicity test (§72-5). Since the fish life-cycle toxicity test was the most similar study type, its Standard Evaluation Procedure (SEP) was used in the evaluation of this study.

Significant guideline deviations included: the study was not conducted in accordance with USEPA GLP provisions; only one concentration level was used, making assessment of endpoints difficult; exposure of the $F_0$-generation fish began when the fish were 8-months old, so that $F_0$-generation embryo and larval/juvenile assessment was not performed; $F_0$-generation fish that died during exposure were replaced with fish from a concurrently-maintained reserve/replacement group; $F_1$-generation fish were maintained for only 6 weeks; the test water was aerated during exposure for all groups; DO was generally maintained at only >60% saturation (despite aeration); aside from being bred in the testing facility, information regarding the source of the $F_0$-generation fish was not provided; lighting conditions during acclimation were not reported; pH was generally lower than recommended for this test species; test water hardness was significantly higher than recommended levels, and alkalinity and conductance were apparently not measured; the size and design of larval chambers did not follow guideline recommendations; and flow rates were significantly higher than recommended rates.

The reproductive behavior of driving females to the brood hole was statistically-reduced ($p \leq 0.05$) compared to controls (55% for controls versus 45% for the test group). The study author reported that since no historical data on this parameter are available, and since only one concentration was used, it is questionable whether this small difference is test substance-related or an arbitrary effect.

Two pair (of 15) from the treatment group exhibited delayed egg production. The study author reported that this cannot be interpreted as a substance-related effect since only one test group is available and the number of pairs is comparably low.

The number of spawns/female was statistically-reduced ($p \leq 0.01$) compared to controls (14.7 for controls versus 6.73 for the test group); however, the number of eggs/spawn was notably higher in the treatment group (65.4 for controls versus 113.7 for the test group). Consequently, the number of eggs/female was only slightly lower in the test group (959 for controls versus 766 for the test group), with no statistical significance. Therefore, the study author questioned the biological significance of this finding.

Hatch survival was statistically-reduced ($p \leq 0.01$) both at the start and end of hatch

compared to controls (at end of hatch, 66.5 for controls versus 42.5 for the test group). Since only four pairs/test group contributed to the production of the $F_1$-generation and since fertility rates (sum of all fertile eggs/female divided by the sum of all eggs/female) in the $F_0$-generation showed high variability, the study author reported that the lower survival rate might be due to the normal variation between pairs and even between the spawnings of one pair. The study author further concluded that the biological relevance of the lower survival until hatch finding cannot be evaluated since only one concentration group was available.

In conclusion, because only one test concentration was used in this study, neither a LOEL nor a definitive NOEL was established.

Statistical verification by the reviewer revealed no differences in sublethal effects or mortality between control and treatment groups. The number of egg clutches was significantly reduced in the treatment group, relative to control. At the beginning of hatch, embryo survival was significantly reduced in the treatment group, but not survival during hatch. At the termination of hatch, larvae survival was reduced significantly. Survival from day 78 to test termination was significantly reduced. However, number of eggs, eggs per clutch, fertility rate, time to hatch, and duration of hatch did not differ significantly. Also, male and female lengths and weights did not differ significantly.

The reviewer's conclusions generally agreed with those of the study author. High variability may have precluded the detection of statistically significant differences. Furthermore, the inclusion of only a single treatment group precluded establishing a clear directional effect of treatment. In summary, a sufficient number of endpoints was reduced to suggest that the treatment concentration adversely affected fathead minnow reproduction.

## 15. REVIEWER'S STATISTICAL RESULTS:

37-03 egg clutches
File: 37-03c Transform: NO TRANSFORMATION

BONFERRONI T-TEST - TABLE 1 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | TRANSFORMED MEAN | CALCULATED IN ORIGINAL UNITS | T STAT | SIG |
|---|---|---|---|---|---|
| 1 | control | 11.063 | 11.063 | | |
| 2 | 0.1 mg/L | 6.313 | 6.313 | 2.060 | * |

Bonferroni T table value = 2.04 (1 Tailed Value, P=0.05, df=31,2)

37-03 egg clutches
File: 37-03c Transform: NO TRANSFORMATION

BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | NUM OF REPS | Minimum Sig Diff (IN ORIG. UNITS) | % of CONTROL | DIFFERENCE FROM CONTROL |
|---|---|---|---|---|---|
| 1 | control | 16 | | | |
| 2 | 0.1 mg/L | 16 | 4.704 | 42.5 | 4.750 |

37-03 eggs
File: 37-03e Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 1 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | TRANSFORMED MEAN | MEAN CALCULATED IN ORIGINAL UNITS | T STAT | SIG |
|---|---|---|---|---|---|
| 1 | control | 958.667 | 958.667 | | |
| 2 | 0.1 mg/L | 765.667 | 765.667 | 0.873 | |

Bonferroni T table value = 2.06 (1 Tailed Value, P=0.05, df=26,2)

37-03 eggs
File: 37-03e Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | NUM OF REPS | Minimum Sig Diff (IN ORIG. UNITS) | % of CONTROL | DIFFERENCE FROM CONTROL |
|---|---|---|---|---|---|
| 1 | control | 12 | | | |
| 2 | 0.1 mg/L | 15 | 454.398 | 47.4 | 193.000 |

37-03 eggs/clutch
File: 37-03p Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 1 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | TRANSFORMED MEAN | MEAN CALCULATED IN ORIGINAL UNITS | T STAT | SIG |
|---|---|---|---|---|---|
| 1 | control | 67.909 | 67.909 | | |
| 2 | 0.1 mg/L | 111.000 | 111.000 | -1.725 | |

Bonferroni T table value = 2.06 (1 Tailed Value, P=0.05, df=24,2)

37-03 eggs/clutch
File: 37-03p Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | NUM OF REPS | Minimum Sig Diff (IN ORIG. UNITS) | % of CONTROL | DIFFERENCE FROM CONTROL |
|---|---|---|---|---|---|
| 1 | control | 11 | | | |
| 2 | 0.1 mg/L | 14 | 51.546 | 75.9 | -43.091 |

37-03 fertility rate
File: 37-03f Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 1 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | TRANSFORMED MEAN | MEAN CALCULATED IN ORIGINAL UNITS | T STAT | SIG |
|---|---|---|---|---|---|
| 1 | control | 57.573 | 57.573 | | |
| 2 | 0.1 mg/L | 54.171 | 54.171 | 0.460 | |

Bonferroni T table value = 2.06 (1 Tailed Value, P=0.05, df=24,2)

37-03 fertility rate
File: 37-03f Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | NUM OF REPS | Minimum Sig Diff (IN ORIG. UNITS) | % of CONTROL | DIFFERENCE FROM CONTROL |
|---|---|---|---|---|---|
| 1 | control | 11 | | | |
| 2 | 0.1 mg/L | 14 | 15.256 | 26.5 | 3.401 |

37-03 weight
File: 37-03w Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 1 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | TRANSFORMED MEAN | MEAN CALCULATED IN ORIGINAL UNITS | T STAT | SIG |
|---|---|---|---|---|---|
| 1 | control | 4.603 | 4.603 | | |
| 2 | 0.1 mg/L | 4.414 | 4.414 | 0.713 | |

Bonferroni T table value = 2.06 (1 Tailed Value, P=0.05, df=26,2)

37-03 weight
File: 37-03w Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | NUM OF REPS | Minimum Sig Diff (IN ORIG. UNITS) | % of CONTROL | DIFFERENCE FROM CONTROL |
|---|---|---|---|---|---|
| 1 | control | 12 | | | |
| 2 | 0.1 mg/L | 15 | 0.546 | 11.9 | 0.189 |

37-03 female weight
File: 37-03fw Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 1 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | TRANSFORMED MEAN | MEAN CALCULATED IN ORIGINAL UNITS | T STAT | SIG |
|---|---|---|---|---|---|
| 1 | control | 2.326 | 2.326 | | |
| 2 | 0.1 mg/L | 2.613 | 2.613 | -1.300 | |

Bonferroni T table value = 2.06 (1 Tailed Value, P=0.05, df=26,2)

37-03 female weight
File: 37-03fw Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | NUM OF REPS | Minimum Sig Diff (IN ORIG. UNITS) | % of CONTROL | DIFFERENCE FROM CONTROL |
|---|---|---|---|---|---|
| 1 | control | 12 | | | |
| 2 | 0.1 mg/L | 15 | 0.455 | 19.5 | -0.288 |

37-03 length (males)
File: 37-03l Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 1 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | TRANSFORMED MEAN | MEAN CALCULATED IN ORIGINAL UNITS | T STAT | SIG |
|---|---|---|---|---|---|
| 1 | control | 6.958 | 6.958 | | |
| 2 | 0.1 mg/L | 6.860 | 6.860 | 0.589 | |
| 3 | junk | 6.500 | 6.500 | 1.391 | |

Bonferroni T table value = 2.06 (1 Tailed Value, P=0.05, df=26,2)

37-03 length (males)
File: 37-03l Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | NUM OF REPS | Minimum Sig Diff (IN ORIG. UNITS) | % of CONTROL | DIFFERENCE FROM CONTROL |
|---|---|---|---|---|---|
| 1 | control | 12 | | | |
| 2 | 0.1 mg/L | 15 | 0.343 | 4.9 | 0.098 |
| 3 | junk | 2 | 0.677 | 9.7 | 0.458 |

37-03 length (females)
File: 37-03lf Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 1 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | TRANSFORMED MEAN | CALCULATED IN ORIGINAL UNITS | T STAT | SIG |
|---|---|---|---|---|---|
| 1 | control | 5.900 | 5.900 | | |
| 2 | 0.1 mg/L | 6.040 | 6.040 | -1.014 | |
| 3 | junk | 6.500 | 6.500 | -2.204 | |

Bonferroni T table value = 2.06 (1 Tailed Value, P=0.05, df=26,2)

37-03 length (females)
File: 37-03lf Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

| GROUP | IDENTIFICATION | NUM OF REPS | Minimum Sig Diff (IN ORIG. UNITS) | % of CONTROL | DIFFERENCE FROM CONTROL |
|---|---|---|---|---|---|
| 1 | control | 12 | | | |
| 2 | 0.1 mg/L | 15 | 0.284 | 4.8 | -0.140 |